# ON FOOD AND FEEDING OF LARVAE OF THE AMERICAN OYSTER, C. VIRGINICA

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Feeding experiments have shown that larvae of the American oyster, *Crassostrea virginica,* are limited as to the types of food they can utilize, by factors other than the size of the micro-organism or particle. We have previously reported four species of marine bacteria and three species of flagellates that had no effect on the rate of growth of oyster larvae (Davis, 1950a). In one experiment, for example, in cultures that received sulfur bacteria as food, the larvae averaged only 94.05 $\mu$ in length at 14 days, a size not significantly different from that of larvae in unfed control cultures. Moreover, it was an increase of only about 20 $\mu$ over the initial size of early straight-hinged larvae which usually measure about 74–76 $\mu$ in length. In a pair of parallel cultures fed mixed phytoplankton composed chiefly of Chlorella, the larvae averaged 140.75 $\mu$ in length by the 14th day or had gained about 65 $\mu$ over their initial size.

The same series of experiments also showed that marine detritus, which we had collected from several sources, was not utilized by the larvae, and that organic media added to the larval cultures caused no increase in the rate of growth of the larvae.

On the basis of the above experiments it was reported (Davis, 1950a) that our mass culture of green phytoplankton consisting chiefly of Chlorella, which we shall refer to as "mixed Chlorella," was the best food for oyster larvae then available. It did not consistently induce a good rate of growth when fed to larvae during their earlier stages, but once the larvae had attained a size of approximately 125 $\mu$ they appeared to utilize the "mixed Chlorella" quite readily.

In the feeding experiments on oyster larvae reported in the following pages, we have tested nine additional species of marine bacteria, isolated from the mud of Milford Harbor by Dr. Burkholder of Yale University; six more species of marine flagellates, including some of the same varieties used by Bruce, Knight and Parke (1940), obtained from Dr. Russell of the Plymouth Laboratory, England; and a bacteria-free culture of *Chlorella* sp. isolated from our mixed phytoplankton culture by Dr. Ralph Lewin of Yale University. These feeding experiments were conducted during the winter and early spring using oysters brought to spawning condition by the method described by Loosanoff (1945) and by Loosanoff and Davis (1952).

To insure uniformity in size, quality, and number of larvae in the different culture jars of a series, at the beginning of an experiment, and except for foods, to provide as nearly equal treatment of all cultures as possible, the following procedure has been made standard practice.

To obtain fertilized eggs, the conditioned oysters were placed in spawning dishes, filled with sea water that had been filtered through cotton to remove debris

and most planktonic forms, and spawning was induced by adding small quantities of sperm suspension and raising the temperature to about 30.0° C. The eggs of *C. virginica,* because of their small size, cannot readily be separated from excess sperm, as can those of *Venus mercenaria* (Loosanoff and Davis, 1950). The spawning females were, therefore, separated from the males and placed in spawning dishes containing only a slight excess of sperm.

The fertilized eggs were collected in a tall narrow jar by screening the contents of the spawning dishes through stainless steel screens having 100 meshes per inch. This screen allowed the fertilized eggs to pass through unharmed but retained larger debris and feces expelled by the oysters during spawning. Since we wished to have 5000 larvae per liter in our experimental cultures, the egg suspension in the tall narrow jar was thoroughly stirred with a perforated plastic plunger to insure uniform distribution of the eggs and a sample was withdrawn and the number of fertilized eggs per ml. determined.

Only rarely did 100 per cent of the fertilized eggs develop into normal straight-hinged veliger larvae, but if less than 50 per cent developed normally, we discarded the larvae. It became our practice, therefore, to introduce enough fertilized eggs into each culture jar to give approximately twice the desired number of larvae if all the eggs should develop normally, *i.e.,* about 10,000 fertilized eggs per liter. The 20-liter earthenware culture jars were then filled with cotton-filtered sea water and the eggs permitted to develop for 48 hours with no supplemental feeding.

After 48 hours the veliger larvae, fully protected by their shells, were collected by passing the contents of each culture jar through a 325-mesh stainless steel screen. The screen retained the shelled veligers but permitted undeveloped eggs, and embryos that had not progressed to the shelled stage, to pass through. The normal larvae from all culture jars were thus collected and pooled in 4 to 7 liters of sea water in a tall narrow jar. After thoroughly mixing the contents of this jar with a perforated plastic plunger, to obtain a uniform distribution of the larvae, a sample was withdrawn and the number of larvae in one ml. of the pooled culture determined. By keeping the pooled culture thoroughly mixed, with the larvae uniformly suspended, and taking appropriate volumes, the desired number of larvae could be introduced into each culture. The larvae of all cultures were, therefore, closely comparable in size and quality, as well as number, when the different food treatments were started on the second day.

All culture jars were kept covered so they were uniformly dark and all were in a common water bath. Although the temperature of the water bath fluctuated between 21.0° and 23.0° C. all cultures were equally affected. The sea water in all cultures was replaced with fresh cotton-filtered sea water every second day (Loosanoff and Davis, 1950).

Neither aeration nor mechanical agitation is necessary for normal development of oyster larvae when the water is changed every second day. We did not, therefore, use either aeration or mechanical agitation in these experiments as it is difficult to insure equality of such treatments in a series of cultures.

Samples were taken whenever desired by first washing the entire contents of a culture jar onto the 325-mesh screen, then re-suspending the larvae in 1000 ml. of sea water in a parallel-sided graduated cylinder. To insure uniform distribution of the larvae, the contents of the cylinder were thoroughly mixed with a per-

forated plastic plunger and a one-, two- or five-cc. sample was withdrawn and preserved. All the larvae in a sample were transferred to a Sedgwick-Rafter cell and examined under a compound microscope. Larvae that were living at the time the sample was taken could thus be identified and counted to determine the per cent surviving and a random sample of 100 of these larvae was measured to obtain growth data.

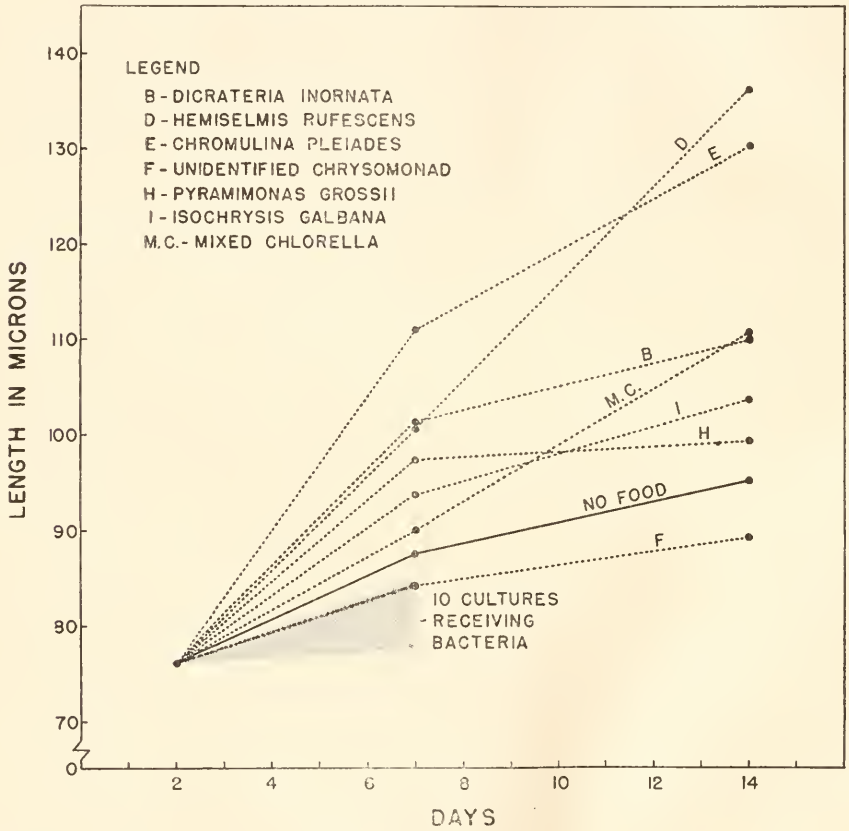


FIGURE 1. Growth of larvae of *C. virginica* fed different foods. Each point on a curve represents the mean length of 100 larvae. Flagellates D, E, and I were fed at the rate of 10,000 per ml./day. Flagellates H and F were fed at the rate of 5000 per ml./day. Flagellate B was fed at the rate of 10,000 per ml./day for first seven days and 5000 per ml./day thereafter.

Eighteen parallel cultures were used in the first of the present series of experiments. Each of the first nine received a different species of the marine bacteria isolated by Dr. Burkholder, six of the remaining each received a different species of the marine flagellates received from England, one received *B. coli* plus a bacteriophage, one received our "mixed Chlorella" and the final culture, which received no supplemental food, served as the control.

The larvae in all ten cultures receiving bacteria and those in the culture receiving flagellate F, an unidentified chrysomonad, grew less rapidly than did those in the unfed control culture (Fig. 1). Moreover, in the ten cultures receiving bacteria, the larvae were all dead by the eleventh day. We can assume that these bacteria and flagellate F were not utilized.

In the remaining six cultures that received supplemental food, the larvae grew more rapidly than did those in the unfed control. We can conclude, therefore,

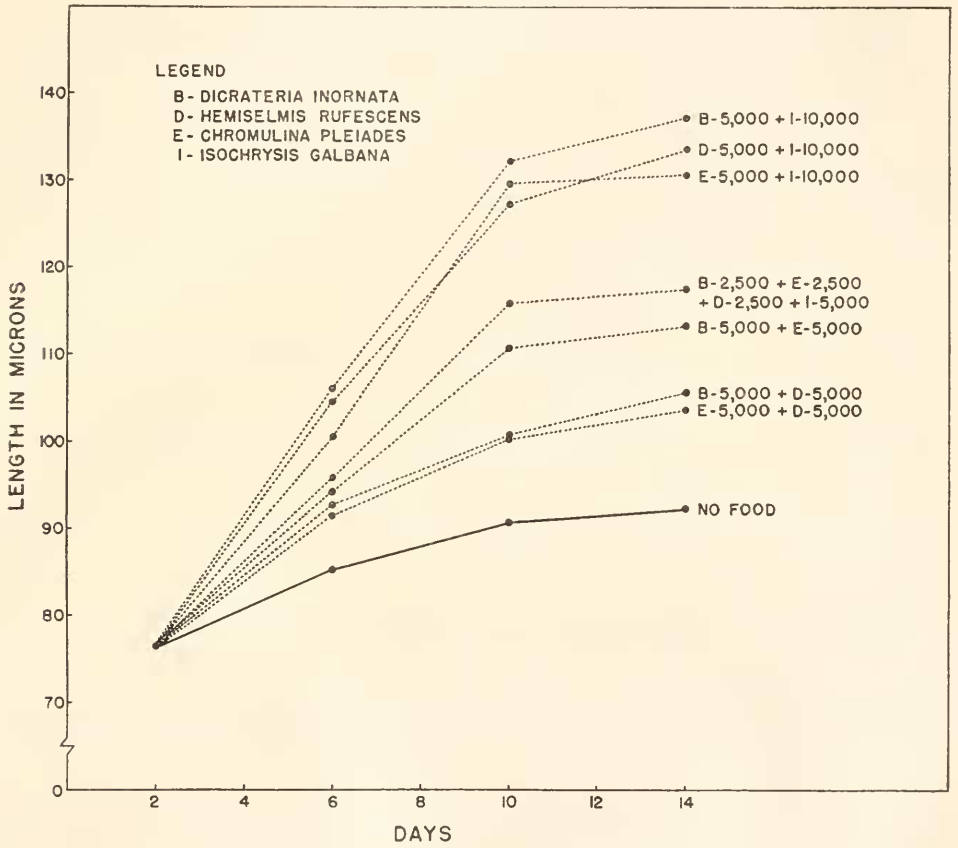


FIGURE 2. Growth of larvae of *C. virginica* fed combinations of flagellates. Each point on a curve represents the mean length of 100 larvae from each of duplicate cultures. Numbers following letter designation of flagellates indicate rate of feeding in number of flagellates per ml./day.

that oyster larvae probably do utilize the five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Hemiselmis rufescens, Isochrysis galbana* and *Pyramimonas grossii,* as well as our "mixed Chlorella."

Unfortunately, due to difficulties in mass production of the flagellates, we were not able to feed equal numbers of each flagellate nor were we able to keep the rate of feeding constant in the larval culture receiving flagellate B (Dicrateria).

In this culture Dicrateria was added at the rate of 10,000 cells per ml./day for the first seven days but after that it was necessary to reduce the rate to 5000 cells per ml./day.

Flagellate I (Isochrysis) and flagellate D (Hemiselmis) were added at the rate of 10,000 cells per ml./day throughout the experiment, while flagellates F (the unidentified chrysomonad) and H (Pyramimonas) were added at the rate

TABLE I

*Mean length of larvae (in microns), receiving different food treatments, at 6, 10 and 14 days with growth increments calculated as mean length minus mean length of 2-day-old larvae (76.5μ)*

| Food treatment | Mean length 6 days | Growth increment 2–6 days | Mean growth increment | Mean length 10 days | Growth increment 2–10 days | Mean growth increment | Mean length 14 days | Growth increment 2–14 days | Mean growth increment |
|---|---|---|---|---|---|---|---|---|---|
| No food | | | | | | | | | |
| Culture No. 1 | 85.90 | 9.40 | | 92.40 | 15.90 | | 93.25 | 16.75 | |
| Culture No. 2 | 84.60 | 8.10 | 8.750 | 89.30 | 12.80 | 14.350 | 91.35 | 14.85 | 15.800 |
| B+E | | | | | | | | | |
| Culture No. 1 | 95.60 | 19.10 | | 112.20 | 35.70 | | 113.35 | 36.85 | |
| Culture No. 2 | 92.75 | 16.75 | 17.925 | 109.35 | 32.85 | 34.275 | 113.30 | 36.80 | 36.825 |
| B+D | | | | | | | | | |
| Culture No. 1 | 93.80 | 17.30 | | 100.60 | 24.10 | | 102.83 | 26.35 | |
| Culture No. 2 | 91.85 | 15.35 | 16.325 | 100.75 | 24.25 | 24.175 | 108.50 | 32.00 | 29.175 |
| B+I | | | | | | | | | |
| Culture No. 1 | 106.65 | 30.15 | | 133.75 | 57.25 | | 139.60 | 63.10 | |
| Culture No. 2 | 105.40 | 28.90 | 29.525 | 130.55 | 54.05 | 55.650 | 134.75 | 58.25 | 60.675 |
| E+D | | | | | | | | | |
| Culture No. 1 | 91.95 | 15.45 | | 99.25 | 22.75 | | 103.70 | 27.20 | |
| Culture No. 2 | 90.85 | 14.35 | 14.900 | 101.70 | 25.20 | 23.975 | 103.95 | 27.45 | 27.325 |
| E+I | | | | | | | | | |
| Culture No. 1 | 98.65 | 22.15 | | 127.10 | 50.60 | | 129.95 | 53.45 | |
| Culture No. 2 | 102.35 | 25.85 | 24.000 | 132.25 | 55.75 | 53.175 | 131.35 | 54.85 | 54.150 |
| D+I | | | | | | | | | |
| Culture No. 1 | 104.30 | 27.80 | | 125.00 | 48.50 | | 131.20 | 54.70 | |
| Culture No. 2 | 104.95 | 28.45 | 28.125 | 129.40 | 52.90 | 50.700 | 135.75 | 59.25 | 56.975 |
| B+E+D+I | | | | | | | | | |
| Culture No. 1 | 94.75 | 18.25 | | 116.05 | 39.55 | | 116.90 | 40.40 | |
| Culture No. 2 | 97.05 | 20.55 | 19.400 | 114.70 | 38.20 | 38.875 | 117.75 | 41.25 | 40.825 |

of 5000 cells per ml./day. Moreover, since this was a preliminary experiment, single cultures were used and the results are consequently less reliable than in later experiments where duplicate or triplicate cultures were used to test each food. Isochrysis, for example, appears to be a comparatively much poorer food here than it proved to be in later experiments. Note that the larvae fed "mixed Chlorella" and those fed flagellate D (Hemiselmis) grew slightly faster after the

seventh day, while those receiving other foods grew somewhat less rapidly the second week (Fig. 1). This may indicate that these two forms are more readily utilized by the larvae in the later stages of development.

Subsequently, in experiments of factorial design, we have fed oyster larvae various combinations of those flagellates which the previous experiment had shown the larvae could utilize. We hoped to estimate the relative value of the different flagellates as foods for oyster larvae, and to determine whether some combination of them might provide a more balanced diet which would result in more rapid larval growth.

The results of a typical experiment of this kind show that certain combinations did appear to give more rapid growth of larvae than others (Fig. 2). The combination of the flagellates B + E, for example, gave more rapid growth than either the combination B + D or E + D. Nevertheless, the greatest differences are correlated with the total number of flagellates given per day. Thus, the three combinations B + I, D + I, or E + I, each consisting of a total of 15,000 flagellates per ml./day gave definitely more rapid growth than did the combination B + E + D + I which consisted of a total of only 12,500 cells per ml./day. More-

TABLE II

*Ratios of the mean growth increments, due to each food combination, to the mean growth increments of larvae in the unfed control cultures. (Standard error of averages 3.75%)*

| Days | No food | B+E | B+D | B+I | E+D | E+I | D+I | B+E+D+I |
|---|---|---|---|---|---|---|---|---|
| 2–6 | 1 | 2.049 | 1.866 | 3.374 | 1.703 | 2.743 | 3.214 | 2.217 |
| 2–10 | 1 | 2.389 | 1.685 | 3.878 | 1.671 | 3.706 | 3.533 | 2.709 |
| 2–14 | 1 | 2.331 | 1.847 | 3.840 | 1.729 | 3.427 | 3.606 | 2.584 |
| Average | 1 | 2.256 | 1.799 | 3.697 | 1.701 | 3.292 | 3.451 | 2.503 |

over, this latter combination in turn gave slightly more rapid growth than did any of those combinations consisting of only 10,000 flagellates per ml./day. Thus, the total number of flagellates added appears to be an important factor and suggests that the different species of flagellates may be of nearly equal value as foods for oyster larvae.

Statistical tests showed that the differences in rate of larval growth, attributable to differences in the total number of flagellates present, were indeed significant. Nevertheless, to account completely for the results observed it is also necessary to assume that the different species of flagellates were not of equal value as foods for oyster larvae.

An analysis of variance of the growth increments (Table I) showed that the average difference between growth increments of duplicate cultures was not significant. We are justified, therefore, in using the ratio of the mean growth increments of the duplicate cultures as a relative measure of the efficiency of the different foods. Dividing the mean growth increments for each period by the mean growth increment of the unfed cultures for the same period gives relatively constant ratios (Table II). An analysis of variance showed that differences between the ratios for the 2–6-, 2–10- and 2–14-day periods were not significant. The average ratio

can be used, therefore, as a numerical estimate of the relative over-all efficiency of the different food combinations at the particular concentrations used in this experiment.

This suggests that if the effect on growth of larvae on any given food really is constant, the uncontrolled factors responsible for the growth of unfed cultures have a multiplicative power on the effect of the different food combinations. If we postulate, in addition, that the effect of each species of flagellate is proportional to its concentration and independent of the presence or absence of other foods, we can formulate the following equation for the growth increment (Y) of any combination of foods: $Y = k\ (1 + b + e + \ldots)$, where k is the uncontrolled variable responsible for the growth of unfed cultures and b, e, . . . are the effects of the foods B, E, etc. Thus, if b is the effect of 2500 *B* (Dicrateria) per cc., e is the effect of 2500 E (Chromulina) per cc., d is the effect of 2500 D (Hemiselmis) per cc. and i is the effect of 5000 I (Isochrysis) per cc., the equation for the growth increment of the food combination B + E + D + I becomes $Y = k\ (1 + b + e + d + i)$, while for the combination B + E, in this experiment it becomes $Y = k\ (1 + 2b + 2e)$ and for the unfed cultures is $Y = k\ (1 + 0)$ or $Y = k$. From the design of the experiment and having a value for k (the observed growth increment of unfed cultures) we can calculate values for b, e, d and i using the values given for the food combinations in the average ratio (Table II). Thus b, the effect of 2500 Dicrateria per cc., becomes: $b = 1/7\{[(2.256–1) + (1.799–1) + (3.697–1) + (2.503–1)] - 3/4[(1.701–1) + (3.292–1) + (3.451–1)]\}$ or $b = 1/7(6.255–4.083) = 0.3103$.

Similarly, the separate effects of the other flagellates become $e = 0.185$, $d = 0.110$, and $i = 0.982$, but this ratio compares 2500 of each B, E and D with 5000 of I. When all are adjusted to the 10,000 level so that we compare the flagellates cell for cell, they become $b = 1.240$, $e = 0.740$, $d = 0.440$ and $i = 1.964$. Since these values were taken from the average ratio of the food combinations, the values represent the over-all efficiency of the foods throughout the experiment.

Similar ratios or estimates of food values can be worked out for the separate four-day periods; corrected by successive approximations and adjusted to the 10,000 level for each flagellate they are:

| | b | e | d | i |
|---|---|---|---|---|
| 2–6 days | 1.372 | 0.788 | 0.776 | 1.716 |
| 6–10 days | 1.616 | 1.112 | −0.820 | 3.226 |
| 10–14 days | 3.776 | −2.504 | 5.123 | 0.450 |

The value of most of the flagellates varies considerably. These variations probably represent day-to-day fluctuations in both physiological state and purity of the flagellate cultures used as foods, for it is known that in many micro-organisms the chemical composition varies with their physiological condition. Thus, although B (Dicrateria), in this experiment, appears to be a better food than E (Chromulina) in approximately 50 per cent of the experiments of this series the reverse is true. It seems probable, therefore, that differences in rate of growth of oyster larvae brought about by variations in the physiological condition of Chromulina and Dicrateria are as great or greater than differences dependent upon which of these two species the oyster larvae are fed.

Flagellate D (Hemiselmis) appears to be the poorest of these four species. However, the high value for the 10–14-day period may indicate, as in the previous experiment, that Hemiselmis is more readily utilized by older larvae. Unfortunately, this flagellate culture was lost and we could not obtain further data on its effect on the rate of growth of oyster larvae.

Bruce, Knight and Parke (1940), in feeding experiments on larvae of the European oyster, *Ostrea edulis,* rated flagellate I as a "good to very good" food while rating flagellate B as "fair." Although their tabulated data seem to indicate that flagellate F was utilized and that flagellate D was not, the authors do not discuss these two flagellates further.

The second part of the problem was to determine whether, by combining the various flagellates, we could provide a diet that would bring about more rapid growth of oyster larvae than could be obtained by feeding an equivalent number of cells of a single species. From a statistical viewpoint, if the separate growth increments due to the different foods, as calculated above, can be combined ac-

TABLE III

*Differences between observed and calculated mean lengths of oyster larvae receiving different foods*

| Foods | 6 days | | | 10 days | | | 14 days | | |
|---|---|---|---|---|---|---|---|---|---|
| | Observed | Calculated | Diff. | Observed | Calculated | Diff. | Observed | Calculated | Diff. |
| No food | 85.2 | 84.8 | −0.4 | 90.9 | 90.9 | 0.0 | 92.3 | 92.3 | 0.0 |
| B+E | 94.2 | 93.7 | −0.5 | 110.2 | 110.3 | +0.1 | 113.3 | 113.2 | −0.1 |
| B+D | 92.8 | 93.7 | +0.9 | 100.7 | 100.7 | 0.0 | 105.7 | 108.8 | +3.1 |
| B+I | 106.0 | 104.7 | −1.3 | 132.2 | 134.3 | +2.1 | 137.2 | 137.1 | −0.1 |
| E+D | 91.4 | 91.3 | −0.1 | 100.5 | 100.6 | +0.1 | 103.8 | 103.9 | +0.1 |
| E+I | 100.5 | 102.3 | +1.8 | 129.7 | 130.2 | +0.5 | 130.7 | 130.0 | −0.7 |
| D+I | 104.6 | 102.2 | −2.4 | 127.2 | 126.1 | −1.1 | 133.5 | 133.2 | −0.3 |
| B+E+D+I | 95.9 | 98.0 | +2.1 | 115.4 | 114.7 | −0.7 | 117.3 | 119.6 | +2.3 |

cording to the formula to give a satisfactory fit to the growth increment observed when these foods are used in combination, we will have no reason to believe that there are interactions. In other words, if the effect of B and the effect of E can be added together as in the formula to give a growth increment that agrees closely with that observed when B and E were fed in combination, we will have no reason to believe that the combination B + E is any better as a food than equivalent quantities of B or E alone.

A statistical analysis of the results of the previous experiment shows no evidence that any of the combinations of flagellates tested results in more rapid growth of oyster larvae than would equivalent amounts of any of the flagellates separately. The observed mean lengths of larvae, receiving combinations of the different flagellates as foods, were compared with the mean length calculated from the values of the separate foods given above as the sum of the growth increment given by the formula plus the mean length of larvae in the unfed control cultures (Table III). At six days the maximum difference between observed and calculated mean length is 2.4 $\mu$, at 10 days it is 2.1 $\mu$, and at 14 days it is 3.1 $\mu$. These differences are of

the same order of magnitude as differences between parallel cultures receiving the same treatment and it can be shown that the fit between calculated and observed mean lengths is satisfactory. Therefore, we have no reason to believe that the foods are not completely additive, and no interaction is indicated. Bruce, Knight and Parke (1940) believed that a combination of the flagellates H and I

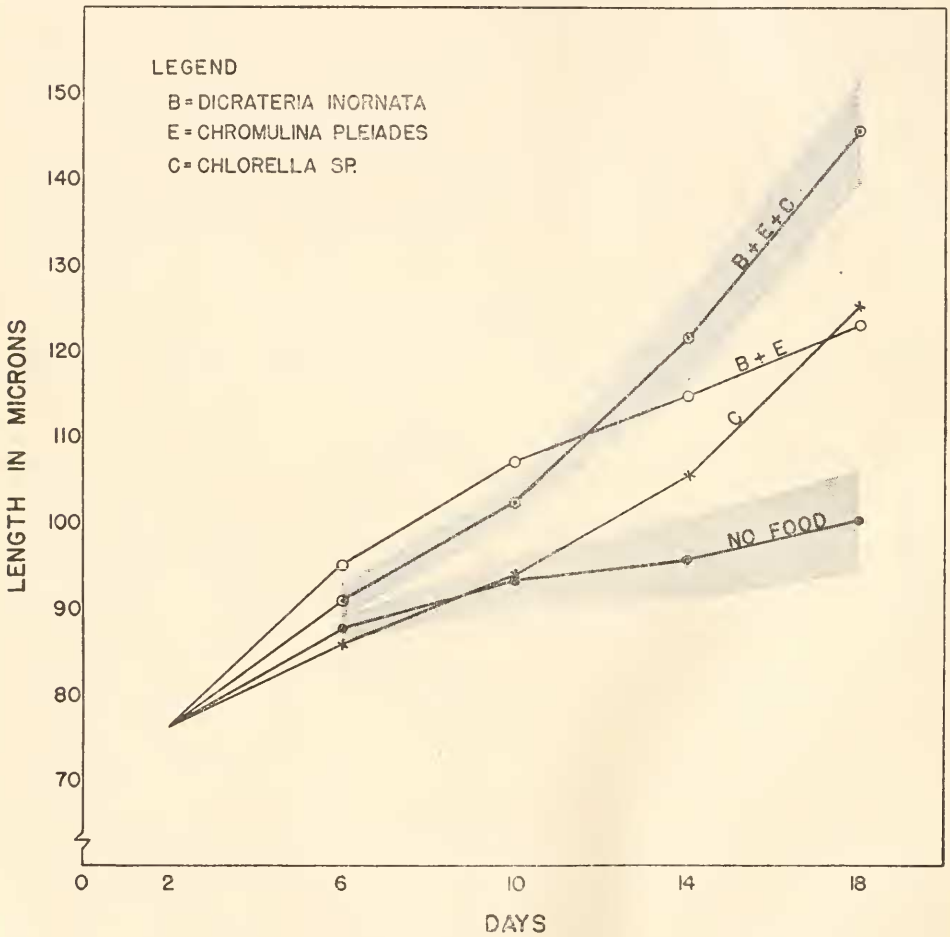


FIGURE 3. Growth of larvae of *C. virginica* fed *Chlorella* sp. alone and in combination with flagellates. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures. Shaded areas represent 95 per cent confidence bands. Flagellates B and E fed at the rate of 5000 per ml./day and *Chlorella* sp. fed at the rate of 50,000 per ml./day.

was especially suitable as food for larvae of *O. edulis* but did not test for an interaction. Thus far we have not tested this combination with larvae of *C. virginica.*

Studies to determine what concentration of any single species of these flagellates would be required to induce the maximum rate of growth of oyster larvae are still

in progress. Preliminary tests indicate, however, with Dicrateria alone as the supplementary food, that this flagellate must be fed at the rate of 25,000 cells per ml./day (the highest rate tested), or higher, to produce the most rapid growth of larvae possible in cultures such as ours, which contain approximately 5000 larvae per liter. Isochrysis has not been tested in varying concentrations, but the larvae grew rapidly when this flagellate was fed at the rate of 20,000 cells per ml./day. When Chromulina was used as the supplemental food, however, the larvae grew slightly faster in cultures receiving 15,000 cells per ml./day than in cultures receiving 20,000 cells per ml./day.

Our experiments indicate, therefore, that while the concentration of flagellates required to give the most rapid growth of larvae probably varies with the species of flagellate, none of the flagellates that were utilizable gave toxic effects when added to our larval cultures at rates up to 15,000 cells per ml./day and for certain species up to 25,000 cells per ml./day. Korringa (1949), on the other hand, although he obtained good growth of larvae and a heavy spatfall of *O. edulis* in his 1947 experiments with concentrations of flagellates between 10,000 and 20,000 per ml., concluded from later experiments that (p. 4) "water initially containing more than 5000 flagellates, or a commensurable great number of other phytoplankton, should be mistrusted as it may contain toxic concentrations of phytoplankton metabolites from the first day the tanks are filled." Imai and Hatanaka (1949) indicate that in culturing larvae of *Crassostrea gigas,* they strive to keep the concentration of the flagellate, *Monas* sp., at a concentration of only 1000 to 2000 per ml. in their cultures. These authors have, at maximum, only about 200 larvae per liter, however, and such low concentrations of flagellates would provide too small a quantity of food to induce appreciable growth of larvae in cultures such as ours with a concentration of approximately 5000 larvae per liter.

In other experiments we sought to determine the effect of the bacteria-free culture of *Chlorella* sp. on the rate of growth of oyster larvae. In these experiments *Chlorella* sp. was tested both alone and in combination with some of the flagellates (Fig. 3). Using triplicate cultures for each treatment, one trio served as a control and received no supplementary food, one trio received *Chlorella* sp. alone (50,000 per ml./day), one trio received a combination of flagellates B + E (5000 per ml./day of each) and one trio received *Chlorella* sp. (50,000 per ml./day) in addition to the combination of flagellates B + E (5000 per ml./day of each).

Our "mixed Chlorella" culture, although apparently being more effectively used by larvae in the later stages of development, had been utilized from the start (Fig. 1). With a pure culture of Chlorella, however, in this and in several repetitions of the experiment, the effect on the growth rate of the larvae during the early stages, although small, is consistently negative (Fig. 3). This is true both when pure Chlorella is added alone, and when it is used in combination with the flagellates. Some time between the sixth and twelfth days, however, the larvae appear to become able to utilize *Chlorella* sp. and it accelerates their growth markedly.

Calculations similar to those previously mentioned give the following values for b, e and c (effect of *Chlorella* sp.) :

| | b | e | c |
|---|---|---|---|
| 2–6 days | 0.252 | 0.329 | −0.164 |
| 6–10 days | 0.191 | 0.579 | 0.323 |
| 10–14 days | 0.598 | 0.580 | 2.364 |
| 14–18 days | 0.620 | 0.630 | 3.467 |

However, when these figures are adjusted to the 10,000 level so that we are comparing food micro-organisms cell for cell, it becomes obvious that Chlorella is inferior to the flagellates as a food on such a basis, thus:

| | b | e | c |
|---|---|---|---|
| 2–6 days | 0.504 | 0.658 | −0.033 |
| 6–10 days | 0.382 | 1.158 | 0.065 |
| 10–14 days | 1.196 | 1.160 | 0.473 |
| 14–18 days | 1.240 | 1.260 | 0.693 |

Again there is no evidence of an interaction of food organisms and the growth increment of the combination B + E + Chlorella is given by $Y = k(1 + b + e + c)$. These experiments explain our previously published observations (Davis, 1950a) that "mixed Chlorella," while not consistently a good food during earlier larval stages, served quite well as food during later larval stages. The good growth obtained during later larval stages is undoubtedly due to the utilization of Chlorella itself by the oyster larvae. The inconsistency of growth of earlier larval stages is understandable since, from these experiments, we know that during these stages the larvae do not utilize Chlorella. Obviously, then, growth during the early larval stages, when "mixed Chlorella" was used as a food, was due to other forms which might or might not be present at any given time in our mass culture of Chlorella, and to small amounts of food in the sea water, which may, as in the experiment shown in Figure 3, carry the larvae through the early stages until they can utilize Chlorella.

Cole (1936) after a review of the literature on the European oyster (*O. edulis*) states that the conclusion that the larvae are able to develop on Chlorella is not supported by results of well designed critical experiments. Presumably the larvae are unable to digest this alga. We have shown here, however, that Chlorella is utilized by older larvae of *C. virginica* and we have unpublished data showing that larvae of *Ostrea lurida*, a species closely related to *O. edulis*, can be reared to metamorphosis on either mixed Chlorella or a bacteria-free culture of Chlorella.

A number of cultures of *C. gigas* have been reared to metamorphosis (Davis, 1950b) using our "mixed Chlorella" as a food, although in general larvae of this species are similar to larvae of *C. virginica* in being limited as to the types of micro-organisms they can utilize as foods. In addition Loosanoff and Davis (1950), Loosanoff, Miller and Smith (1951), and Loosanoff and Marak (1951) have reared *O. edulis, Venus mercenaria, Mya arenaria* and several other species of lamellibranch larvae using our "mixed Chlorella" as the chief food. Such results certainly indicate that the ability to utilize Chlorella as a food is common to several species of lamellibranch larvae.

In the course of our experiments certain differences in rate of growth of oyster larvae, in different members of a trio or pair of cultures receiving the same treatment, appeared to be due to differences in the number of larvae present in the culture. An experiment in which triplicate cultures at each of four different con-

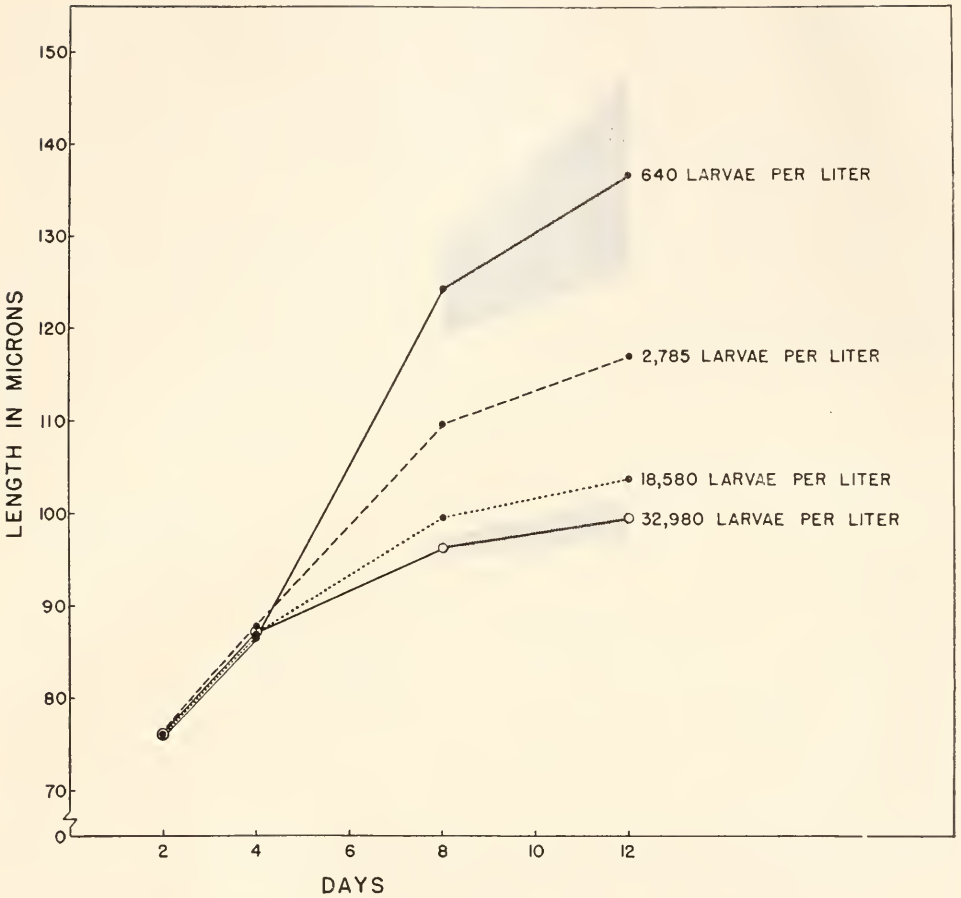


FIGURE 4. Growth of larvae of *C. virginica* cultured at four different concentrations with food constant at 50,000 cells per ml./day. Shaded areas represent 95 per cent confidence bands. Each point on a curve represents the mean length of 100 larvae from each of triplicate cultures.

centrations of larvae were used indicated an inverse relation between the concentration of larvae and their rates of growth (Fig. 4). These cultures all received 50,000 cells per ml./day of our "mixed Chlorella" as a supplementary food.

The between-culture variations, at the two lower concentrations of larvae, were abnormally great, while at the higher concentrations the between-culture variations were normal. Thus at 14 days the average sizes of larvae in the various cultures were as follows:

| Number of larvae per liter | Culture number 1 | Culture number 2 | Culture number 3 |
|---|---|---|---|
| 640 | 140.0 | 140.1 | 125.17 |
| 2,785 | 110.50 | 111.57 | 126.55 |
| 18,580 | 102.65 | 103.35 | 105.35 |
| 32,980 | 98.35 | 98.70 | 101.60 |

The 95 per cent confidence bands (Fig. 4) were calculated with the inconsistent values included. We are probably justified in concluding that with all cultures receiving equal quantities of this food, there is an inverse relation between the concentration of larvae in a culture and their rate of growth, at least after the eighth day. The failure of the inverse relation to appear earlier is probably due to the inability of oyster larvae to utilize the "mixed Chlorella" readily during the earlier stages of development.

We do not know whether similar results would be obtained with other foods, but suspect that they would be, nor do we know what results would be obtained if the quantity of food were kept proportional to the concentration of larvae. The results of the experiment suggest, however, that the concentration of larvae in a culture must be considered in comparing rates of larval growth.

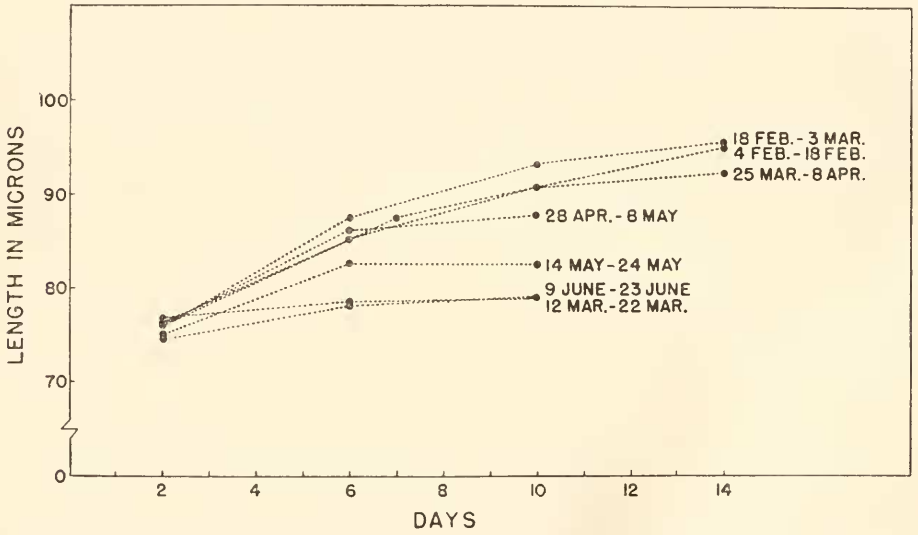


FIGURE 5. Growth curves of unfed control cultures of *C. virginica* larvae showing variations between seven successive experiments. Each point on a curve represents the mean length of 100 larvae from each of duplicate or triplicate cultures.

Other differences, which make comparison of rates of larval growth difficult, are those between successive cultures in which the larvae receive identical treatments. The factor or factors (k) responsible for these differences affect not only the rate of growth of larvae but also, in some experiments, their survival as well. The growth curves of the larvae in the unfed control cultures of seven successive experiments illustrate these differences (Fig. 5). In the 1952 experiments of February 18–March 8 and of February 4–18, the curves appear normal since growth of the larvae in the unfed cultures, although slow, was continuous throughout the 14 days of the experiments. The growth of larvae appears subnormal throughout the May 14–24 experiment, when compared to the above curves, and in the experiments of March 12–22 and June 9–19 almost no growth occurred in these unfed cultures. In the experiment of March 25–April 8 the flattening of

the curve between the tenth and fourteenth days, which reflects an almost complete lack of growth of the larvae during this period, appears abnormal. In the experiments of April 28–May 8 and May 14–24, similar abnormal flattening of the growth curves occurred between the sixth and tenth days.

In each of the three experiments in which the flattening of the growth curves of the unfed cultures occurred after the sixth day, there were also seven pairs of cultures receiving different foods, yet all cultures showed similar flattening of their growth curves simultaneously.

This flattening of growth curves cannot be considered a normal deceleration of growth since it is not correlated with either age of larvae (Fig. 5), or with size (Figs. 2 and 5). Moreover, a pronounced simultaneous upswing of growth curves in all cultures was noted in one experiment and minor simultaneous upward trends were noted in several others. Such simultaneous changes in the growth curves of the larvae in all cultures of an experiment, regardless of food treatment, must be a reflection of variation in some unknown factor or factors common to all cultures. The factors common to all cultures, and least susceptible to control, are the physical, chemical and microbiological constituents of the sea water in which the larvae were reared.

Also strongly suggestive of a variation in the physical or chemical constituents of the sea water was the variation in peak densities of flagellate cultures grown in media prepared from sterilized sea water enriched with constant amounts of nutrient salts. Culture media prepared from sea water taken during periods of poor larval growth gave lower peak densities of flagellates than did media made up from sea water taken during periods of good larval growth.

In all of our experiments, in cultures that received flagellates known to be utilizable, the larvae have grown more rapidly than those in the parallel unfed control cultures. Yet we have not been able to overcome completely the effects of the unknown factor (or factors) by supplemental feeding. For example, under the conditions existing during the May 14–24 and June 9–19 experiments, feeding a combination of the flagellates Isochrysis and Dicrateria produced only about 1/5 as much growth of larvae, in ten days, as did identical concentrations of the same combination of flagellates in the experiment of March 25–April 8. This suggests that one phase of the action of the unknown factor may be to affect the ability of the larvae to utilize the food that is available.

Several authors have suspected unidentified variations in sea water of affecting their results. Loosanoff, Miller and Smith (1951) noted a lack of uniformity of results in consecutive experiments with larvae of *Venus mercenaria* and considered it possible (p. 75) "that at different times the water itself contained certain dissolved substances which, in a manner not yet understood, affected the rate of development of bivalve larvae." Wilson (1951), working in England with polychaete larvae, found a difference between sea water from two different areas and attributed the poor growth of larvae in water from one of these sources to a lack of (p. 18) "some unknown constituent, essential for healthy development" of the species of polychaetes he used. One of these unidentified variants may be that described by Collier, Ray and Magnitzky (1950) who reported a substance in sea water that can be measured photometrically with N-ethyl carbazole, the concentration of which could be correlated with the pumping rate of oysters.

Wangersky (1952) reported that the substance, measured photometrically with N-ethyl carbazole, was a mixture of dehydroascorbic acid and a rhamnoside. He concludes (p. 685) "that the vitamin is present in the sea largely in the form of dehydroascorbic acid." Although we have not yet tried dehydroascorbic acid, we have added ascorbic acid to the sea water in which the larvae were reared. This did not improve the rate of growth of oyster larvae, but merely resulted in a dense growth of bacteria which killed the larvae. Similar exploratory experiments indicate that additions of Vitamin $B_{12}$, iodine, $Mn^{++}$, $Fe^{+++}$, $Zn^{++}$ or $PO_4^{---}$, likewise, do not improve the rate of growth of oyster larvae.

To verify that deleterious changes in the sea water in our laboratory system were not causing the poor growth and high mortalities of our June cultures, an experiment was designed to compare the laboratory sea water with sea water taken directly from Milford Harbor in enamel buckets. Parallel cultures of oyster larvae were started, two in sea water from each source. In addition a single culture of larvae of *Venus mercenaria* was started in laboratory sea water at the same time. All cultures received approximately 50,000 cells per ml./day of the "mixed Chlorella" as food.

At 14 days all the oyster larvae in sea water from both sources were dead, while the Venus larvae still appeared healthy and were growing normally. At 18 days the Venus larvae had reached setting size and a count revealed that approximately 55 per cent of the larvae that had been counted 12 days earlier while still in the straight hinge stage were still living. Thus, although growth was somewhat slower than average (Loosanoff and Davis, 1950; Loosanoff, Miller and Smith, 1951) the Venus larvae had lived and grown to setting stage, as in several previous experiments (Loosanoff, 1950), under conditions in which the oyster larvae had all died.

In other experiments water from points several miles offshore in Long Island Sound was used but again no significant difference in rate of growth of oyster larvae was noted. This experiment indicated that the "water factor" that resulted in poor larval growth was not just a local condition. We agree, therefore, with Wilson (1951), who concluded (p. 18) "it is evident that many animals find no difficulty in living and reproducing under water conditions that seem to affect some other species adversely."

We do not yet know what this "water condition" is, nor whether it is the presence of some inhibitory or toxic substance that causes poor growth, or, as Wilson (1951) believes, it is the lack of something necessary for good growth. Our experiments do indicate that one phase of its action is to affect the ability of larvae of *C. virginica* to utilize the food that is present.

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## Summary

1. None of the 13 species of marine bacteria tested to date was utilized as food by oyster larvae.

2. Five species of flagellates, *Dicrateria inornata, Chromulina pleiades, Isochrysis galbana, Hemiselmis rufescens* and *Pyramimonas grossii,* were utilized as food by oyster larvae, while another, an unclassified chrysomonad, in addition to the three flagellates previously reported, was not.

3. *Chlorella* sp. was not utilized as food by young oyster larvae but was utilized during later larval stages.

4. None of the combinations of foods tried gave any evidence of providing a more balanced diet or more rapid larval growth than could be obtained by feeding equivalent quantities of a single food. The effects of all foods tested, including Chlorella, are additive.

5. When equal numbers of cells are fed, different species of flagellates induce different rates of growth of oyster larvae.

6. Species of flagellates also differ in the number of cells needed to induce the maximum rate of growth of oyster larvae.

7. The maximum concentration of food organisms that can be created in water containing oyster larvae without unfavorably affecting the larvae varies with the species.

8. With the number of food organisms equal, the rate of growth of oyster larvae had an inverse relation to the number of larvae per unit volume.

9. Variations between rates of growth of larvae in cultures receiving the same treatment in successive experiments appear to be due to some variable factor in the sea water that affects the ability of the larvae to utilize the food that is present.

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